QUANTITATION AND CHARACTERIZATION OF CHROMOSOMAL ABNORMALITIES IN FRIEND VIRUS LEUKEMIA

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by

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INTRODUCTION

The Friend Virus and Friend Disease

During the examination of Ehrlich's ascites tumor preparations with the electron microscope, Selby et al., 1954, noted that the cytoplasm of the tumor cells contained particles of constant diameter in close array. The close similarity of these particles to virus particles prompted Charlotte Friend in 1957 to search for a viral agent causing the Ehrlich's carcinoma. Her attempts to pass the ascites tumor by a subcutaneous injection of a cell-free extract revealed no evidence of the solid carcinoma in microscopic examination of mice after a fourteen month period. However, at autopsy, six of the thirty mice had enlarged spleens and livers. Cell-free preparations from the spleens and livers of these mice could be transmitted to other mice and induce disease symptoms characteristic of a leukemia.

Friend virus disease is characterized by a marked proliferation of immature mononuclear cells which invade the spleen, liver, bone marrow, kidney, lung, and appear in the peripheral blood. The primary cell type involved (i.e. the neoplastic cell) is an erythrocyte precursor, developing from a primitive reticulum cell to an eyrthroblast cell (Siegler and Rich, 1964, 1966). These neoplastic cells can be seen in various stages of mitosis in the organs they infiltrate. At the terminal stages of the disease, the mice have greatly elevated white blood counts, are anemic and

have pronounced hepatomegaly and splenomegaly. The pathogenesis of Friend virus leukemia was studied in detail by Metcalf, Furth, and Buffet (1959). Their data showed that the proliferation of reticulum cells in the spleen was the first sign of the disease and could be demonstrated histologically three days after infection. They further concluded that the disease was a virus-induced leukemia characterized by independent proliferation of erythroblasts and reticulum cells.

Further studies on the pathogenesis of Friend virus leukemia by Dawson, et al. (1962), centered on the early pathological changes in the spleen. Within a few days after infection, minute rounded foci of actively dividing, small reticulum cells were observed beneath the capsule or adjacent to the trabuculae. Later, slight non-specific increases in the erythromyeloid elements of the spleen were observed. In the early stages the disease was confined to the red pulp of the spleen, but later, the lymphoid elements (white pulp), as well as the red pulp, were replaced by solid masses of actively dividing reticulum cells. Small foci of reticulum cells could occasionally be found in the bone marrow. During the third month, the sinusoids of the liver were infiltrated. The time period of reticulum cell infiltration into the various tissues and organs varies according to the number of virus particles injected (Fieldsteel, et al., 1961). The peripheral blood of these animals showed an anemia and leukocytosis comprised chiefly of lymphocyte-like cells.

In addition, up to 10% of abnormal mononuclear cells were present. When the leukocyte count exceeded 100,000/mm^3, 10-20 per cent of these cells were found to be "Friend cells" (Metcalf, et al., 1959). Characteristically, the Friend cell varies in size from that of a large lymphocyte or a monocyte with a relatively large nucleus, moderate amount of cytoplasm and mitochondrial rods about the nucleus. The hematological classification and origin of this cell is at present obscure, but Boiron, et al., (1965) states that the cell is probably a proerythroblast.

The Friend virus can be transmitted to adult Swiss mice or DBA/2 mice as demonstrated by Friend (1957) through various routes of virus administration: intraperitoneally, subcutaneously, intracerebrally and intramuscularly. Fieldsteel, et al., (1962) carried out serial passages of Friend virus infected splenic filtrates through inbred BALB/c mice and showed that the disease could be induced in these mice resulting in most of the same disease parameters as originally described by Friend in random bred Swiss mice. Further studies by Mirand, et al., (1968) have shown that the splenomegaly caused by Friend virus can be broken down to at least two distinct viruses of the Friend virus complex: spleen-focus forming virus (SFFO) and a lymphatic leukemia virus (LLV). However, no proof can be offered to demonstrate that the SFFO can express by itself the disease symptoms without the presence of LLV.

Two distinct types of Friend virus particles exist. de Harven and Friend (1965) discuss purified virions in Friend leukemia consisting mostly of type C particles, with only 2% to 4% of type Al particles. Type Al particles are slightly less than 100 mμ in diameter and are characterized by an electron-lucent center circled by a dense inner shell of approximately 50 mμ in diameter. Type C particles, which are predominant in most electron micrograph examinations of Friend virus preparations, are 100 mμ in diameter, in which the inner core represents the maximum density. The biological activity of the Al particles still remains a matter of discussion. They have been frequently considered as a younger form of the virus but not necessarily immature (de Harven, 1962). However, the C particles have been hypothetically considered as activated virions (de Harven and Friend, 1965), although Dalton, et al., (1961) considers them to be the infective particles. Yumoto et al. (1965) observed numerous immature and mature virus particles in the spleens of Friend and Rauscher virus-infected mice. The development of these virus particles was characterized by a budding phenomenon from the plasma membrane of lymphoid, erythoid, and reticuloendothelial cells. de Harven and Friend (1965) noted that from the fourth day on, budding viruses could be observed almost everywhere and particularly in the megakaryocytes, platelets, spleen, and thymuses of Friend virus-inoculated mice. However, the budding phenomena in those cells and organs seemed to accompany the development

of the viremia rather than to initiate it. de Harven and Friend (1960) state that the budding phenomenon illustrates a transitional phase in the virus cycle, leading to a mature infective particle previously observed in infective pellets prepared from cell-free filtrates of leukemia tissues. A cytological examination revealed intimate contact of the viruses with Leukemic-cell membranes and thus initiated an hypothesized sequence of the budding phenomenon.

Various chemical and physical tests were carried out to check the stability of the virus by Friend (1957). X-ray treatment with 50,000 r., Lyophilization and storage at 70°C for one year did not appreciably diminish the viruses activity; whereas, ether treatment or heating for 30 minutes at 56°C produced complete inactivation.

Chromosome Aberrations and Tumor Cell Relation

Since the speculations of Boveri in 1914 on the association between chromosome alteration and neoplasm, chromosome aberrations in tumors of a variety of species, including man, have been repeatedly reported. The suitability of leukemia as a model system stimulated numerous attempts to detect chromosome abnormalities in human leukemia as well as the disease induced by viral, chemical, and physical agents in experimental animals.

The pertinence of chromosome studies to experimental oncology has been generally accepted. However, despite considerable effort, the question whether the observed

chromosome changes cause or result from neoplastic growth remains unanswered. Proof for a causative hypothesis is that chromosomal changes inducing the disease would show that the induced aberrations were present during the earliest preneoplastic transformation stages. On the other hand, proof of a result hypothesis would show that the induced chromosome aberrations occurred only after the disease was already established. The virus-induced murine leukemias offer a convenient model system for a test of the causative hypothesis. The system provides a transition from the normal to the neoplastic state under controlled conditions with a reproducible interval between inoculations and the appearance of histologically recognizable tumors.

In a study of six different virus tumor systems in mice and rats, Bayreuther (1960) showed that 98% of the primary and secondary tumor sites in the initial host had a normal chromosome complement. Three out of five virus-induced lines (both solid and ascites) that were transplanted showed the normal species-specific and sex-specific chromosome complement. Bayreuther thereby concluded it is unlikely that tumor viruses cause neoplastic transformation via a chromosome mutation. Wakonig-Vaartaja (1960) found a predominance of the diploid number of chromosome in metaphase plates of diseased mouse spleens during S37 virus leukemia. One of three cases of Friend's leukemia demonstrated significant aneuploidy and three of twenty-two cases of S37 also demonstrated aneuploidy. Wakonig-Vaartaja concluded the

predominant occurrence of the normal diploid number suggested that the aneuploidy is not the primary cause of neoplasia in these leukemias. He further considered these aneuploid cases as secondary evolution thus an effect of the disease and not the cause of it.

Tsuchida and Rich (1964) inoculated HaICR Swiss mice with Friend and Rauscher viruses and within 2 to 4 weeks noted hyperplasia of the erythroid elements of the spleen with resultant splenomegaly. Animals that survived developed leukemia. To distinguish between chromosomal changes during the erythroid period and the leukemic period of hyperplasia, chromosome analyses were done on animals early and late in the course of the disease. The chromosome number of spleen cells in animals 7 to 41 days after inoculation had minimal variation about the normal diploid mode of 40. However, the late disease groups (50-65 days) demonstrated chromosome numbers different from that of the controls. Nine per cent of 147 cells examined showed a mode of 41 as opposed to the normal number. The additional chromosome was observed as a small chromosome in hyperdiploid cell or a metacentric chromosome in other cells. Although the additional chromosome was observed as a small chromosome or a metacentric chromosome in some cells, no conclusive evidence is available for the appearance of the extra chromosome in most of the other hetereodiploid cells with a mode of 41.

Although the mouse with its telocentric chromosomes ranging from 2 to 7 microns is not ideally suited for karotype

analysis, specific chromosome changes can be associated with Friend leukemia virus infection. Ohno and Hauschka (1960) observed that approximately 70 to 80 per cent of Friend virus-infected hyperplastic spleen cells were diploid. Hauschka in a later study (1961) states that the tempting generalization that all virus tumors are diploid seems premature and that there are indications of alterations in chromosomes caused by virus-induced murine leukemias that may depend upon the developmental stage of the disease at the time of observation. In the early splenic enlargement period (2 to 4 weeks after inoculation with a 10^{-3} dilution of Friend virus) achromatic zones or secondary constrictions were observed on certain chromosomes near the centromere by Tsuchida and Rich (1964). During the 2 to 4 weeks after inoculation, severe hyperplasia of intense cellular proliferation developed coincidentally with an increase in number of chromosomes with these secondary constrictions. In the later leukemia period, the aberrations included chromatid breaks, acentric, metacentric, small and giant chromosomes in addition to the secondary constrictions noted in the early splenic enlargement period. However, no specific pattern of appearance could be shown with these new aberrations with the exception of their absence in the preleukemic period.

To elucidate the relationship between the Friend virus and the appearance of chromosomal abnormalities, this investigation was undertaken to quantitate and characterize the induced chromosome changes during the progression of the

Friend virus disease. The objectives of this study were to statistically analyze changes involving (1) the dilution of the Friend virus versus the chromosome number, (2) the dilution of the Friend virus versus the induced aberrations, (3) the chromosome number versus the time sequence of the Friend virus disease, and (4) the dilution of Friend virus and induced abnormalities versus the disease process. The working hypothesis of this study is that the quantitation and characterizations of the chromosomal number and induced aberrations at regular intervals of time during the Friend virus disease would introduce clarification as to whether the changes involved (altered chromosome number and/or induced aberrations) cause or result from the leukemic state of infected animals.

MATERIALS AND METHODS

The stock of Friend virus was supplied by Dr. Stephen Elliott of Drake University. The original stock came from the University of Arizona and was stored at -70°C at Drake University. It was received as a 20% suspension of infected BALB/c mice spleens in sucrose stabilizer and had a titer of $10^{-4.0}ID_{50}$/0.2ml determined by extinction dilution bioassay (Elliott et al., 1970). The undiluted stock pool was thawed in a 37°C water bath, transferred to a centrifuge tube, and spun in a refrigerated centrifuge at 2,000 X G for 10 minutes. The supernatant was drawn off, measured with a pipette and

placed in a sterile test tube. An equal volume of sucrose stabilizer was added to make a 10^{-1} dilution of virus. From this dilution, 0.1 ml was drawn off and added to 9.9 ml of stabilizer to make a 10^{-3} dilution of virus. One tenth milliliter of this dilution was drawn off and added to 9.9 ml to make a 10^{-5} dilution of virus. In all steps the dilutions were mixed thoroughly before use and sterility precautions maintained. All material was maintained in an ice bath during the preparations of virus dilutions and before injection.

Female BALB/c mice were obtained from Cumberland View Farms, Clinton, Tennessee. The mice were kept in groups of 6 to 8 during the experimental period and given food and water ad libitum. To facilitate handling of the number of animals and slides used, the experiment was conducted in two parts. Weeks 3, 4, and 5 were conducted with one group of animals and later, weeks 1 and 2 were completed.

On day zero, mice received intraperitoneally the prepared viral dilutions in 0.2 ml amounts. Thus, 30 animals received 0.2 ml of 10^{-1} virus dilution, 30 animals received 0.2 ml of 10^{-3} virus dilution, and 30 animals received 0.2 ml of 10^{-5} virus dilution. Six mice in each of the virus dilutions were sacrificed each week for spleen cell chromosome analysis. An additional six untreated mice served as uninfected controls.

Each mouse, except three of the control mice, received 0.75 ml of 0.2% colchicine (Ciba Corporation, Summit, New

Jersey) in slightly acidified distilled water before being sacrificed. Ninety minutes later, the mice were sacrificed by cervical dislocation. The animals were quickly weighed, the spleen aseptically removed, weighed, and placed in individual sterile petri dishes with approximately one ml of sterile tissue culture medium #199, pH 6.8-7.2. Three control mice received no colchicine to ascertain if this pretreatment had an effect on the chromosome number or aberration.

Chromosome preparations were prepared by a modification of the method of Fox and Zeiss (1961). Spleens were initially minced in the sterile tissue culture medium using a sterile scapel and a syringe equipped with an 18 gauge needle. Cells were then further separated by aspiration approximately ten times before being transferred to a 15 ml centrifuge tube. Twelve ml of 1% sodium citrate was then added. This suspension of cells and sodium citrate was mixed well, incubated for 30 minutes at 37°C followed by centrifugation at 2,000 X G for 5 minutes. Following this centrifugation, care was taken to not disturb the cell pellet during the following steps. The initial supernatant fluid was discarded and five ml of 3:1 acetic alcohol (3 parts of 95% ethonol, 1 part glacial acetic acid) added. After five minutes, the acetic alcohol was removed, 10 ml of fresh 3:1 acetic alcohol added and the pellets incubated for one hour at 4°C. This 3:1 acetic alcohol was removed after one hour, 10 ml of 45% acetic acid added and allowed to stand 18-24 hours at 4°C. The

following day, the 45% acetic acid was removed and 10 ml of 60% acetic acid added for one hour at 4°C. After this time period, all but 1 ml of the 60% acetic acid was removed. The pellet of cells was then thoroughly suspended in the remaining 60% acetic acid.

A total of 6-10 slides were made from this final suspension of isolated spleen cells for each infected and noninfected animal. Glass slides were placed on the surface of a block of dry ice until a frozen condensate appeared on the surface. The slide was then removed and warmed until the peripheral edges thawed, at which time a drop of cell suspension was placed on the remaining frozen surface. The suspension was immediately dried by gently heating over an alcohol flame.

The chromosome preparations were then stained prior to microscopic examination. The slides were placed for 10 minutes in synthetic aceto-orcein (Esbe Laboratories, Toronto, Canada), rinsed in 45% acetic acid for 2 minutes and progressively dehydrated for 2 minutes respectively in two 95% ethanol and one absolute ethanol solutions. A two minute wash in absolute tertiary-butyl alcohol followed before transfer to a final absolute tertiary-butyl alcohol solution. The slides were made permanent using Euparol vert as a mounting medium.

The stained and mounted slides were initially examined by phase contrast microscopy using a Zeiss Photomicroscope. Slides from each animal were scanned under low objectives

and 30 suitable metaphase figures found for each animal. The chromosome number and the type of abnormalities, if any, on these chromosomes were then determined under oil immersion. In several cases, it was impossible to find and count 30 metaphase figures, and therefore, the total number counted was smaller. Questionable figures observed under oil immersion following scanning were either discarded or examined independently by two people. Analysis of cells once chosen under oil immersion were not rejected because of chromosome number, size, shape, or degree of staining. Representative figures demonstrating normal and abnormal chromosomes and different numbers were photographed using Kodak High Contrast Copy film.

Due to the nature of the data, it was impossible to analyze all at one time; it was therefore broken down into blocks of information based on the objectives sought and prepared for computerized statistical analysis. Tests for analysis of variance were carried out in the categories of spleen weights, number of constrictions and proportion of diploid cells among those cells examined in relation to the dose of virus, time and their possible interaction. An analysis correlation was performed on spleen weights and the average number of constrictions present in mitotic figures demonstrating constrictions. The correlation between spleen weights and the proportion of normal diploid cell patterns of mitotic figures were also examined. The data collected on polyploidy was treated as a frequency distribution based

upon the number of cells exhibiting multiples of 40, per total number of metaphase figures examined. All tests for statistical analysis were carried out at either the 95% or 99% confidence limits.

RESULTS

All mice infected with Friend leukemia virus exhibited hyperplasia of the erythyroid elements of the spleen with a resultant splenomegaly that increased progressively during the five-week course of study. These spleens were analyzed to determine the chromosome changes during the five-week study with 10^{-1}, 10^{-3}, and 10^{-5} dilutions of a Friend virus suspension having a titer of $10^{-4.0}ID_{50}/0.2$ ml.

Chromosome Numbers

Polyploid cells, those cells in metaphase demonstrating a chromosome number in multiples of the diploid number of 40, were shown during the course of infection in BALB/c mice with all three doses of Friend virus. Microscopic examination of spleens from infected animals demonstrated chromosome numbers of 80 (Plate 1a) or higher multiples of 40 which were uncountable (Plate 1b). The frequency of polyploidy versus the diploid number showed no dependency of the observed polyploid state upon dose, or week, or their combined interaction. However, in considering polyploidy as a binomial function, the polyploidy occurring in the 10^{-1}

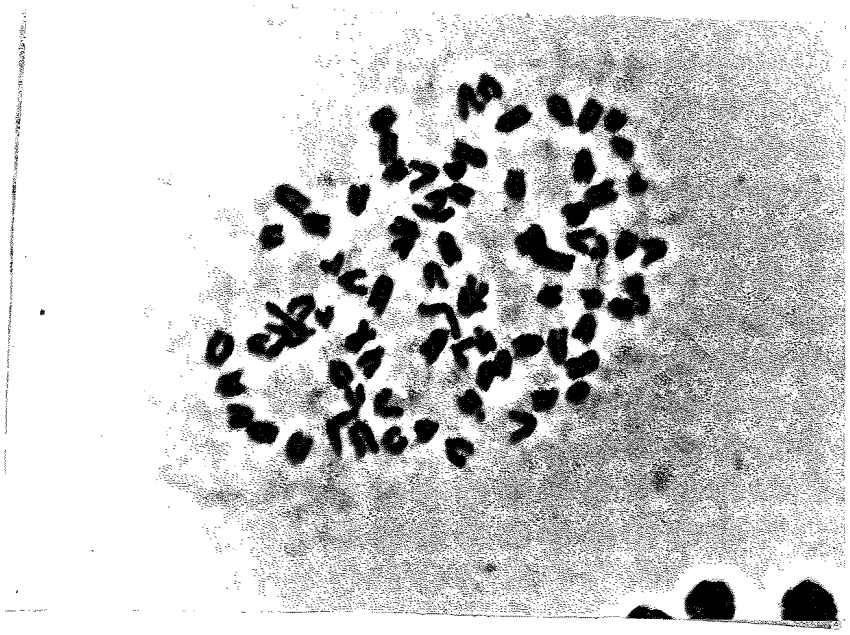


Plate 1a. Polyploid metaphase figure of a spleen cell with 80 chromosomes found in week 1 of a BALB/c mouse that received a 10^{-1} Friend virus dose.

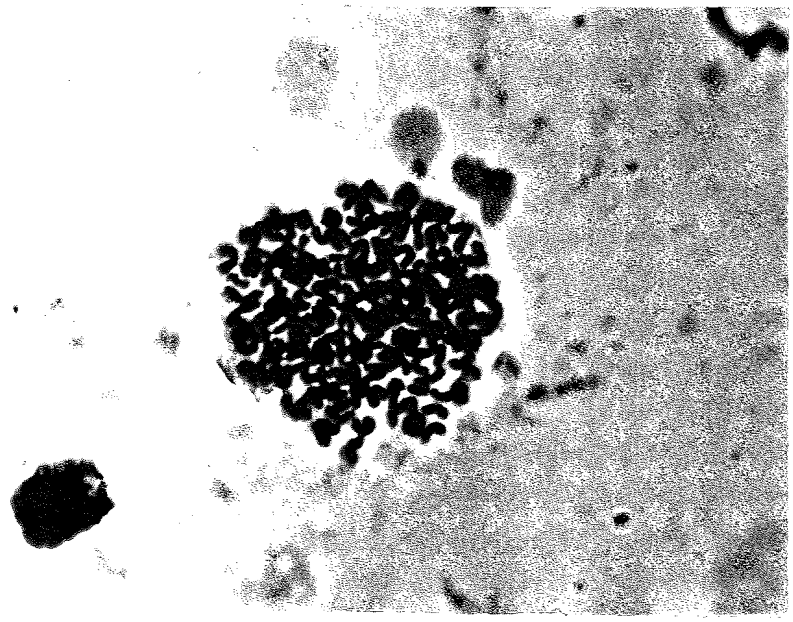


Plate 1b. Polyploid metaphase figure of a spleen cell with an uncountable number of chromosomes found in week 4 of a BALB/c mouse that received a 10^{-5} virus dose.

dilution of Friend virus differed significantly at the 95% confidence limits in weeks 2 and 4 from week 1 (Figure 1). Only a small overlap occurred at week 3. The occurrence of polyploidy among spleen cells in animals at the other virus dilutions was too low to add anything significant in forming trends during the sequence of the disease at these doses. As shown in Figure 1, week 5 was not calculated and week 3 had approximately half as many total cells counted. In addition to the loss of animals in the 10^{-1} dilution in week 3, it was difficult to find mitotic figures in those animals that survived (Table 3).

Minimal variation around the normal diploid number of 40 was observed in examination of the infected spleen cells. Of 2,150 total metaphase figures examined, 21 or 0.9% of these exhibited a mode of 41. Plates 2a and 2b demonstrate metaphase figures with 41 chromosomes. Few cells fell between the hyperdiploid mode of 41 and the multiples of diploid or the polyploid state. However, Plate 3 demonstrates an abnormal number of 49, while two other cells exhibited numbers of 42 and 44. Again, there was no apparent structural difference in the chromosome appearance of the entire set. In many instances, a lower number of chromosomes was noted than the diploid number of 40. Plate 4 shows a typical cell in metaphase demonstrating a chromosome number of 39.

Tables 1, 2 and 3 summarize the distribution of metaphase chromosome numbers per cell in animals receiving virus

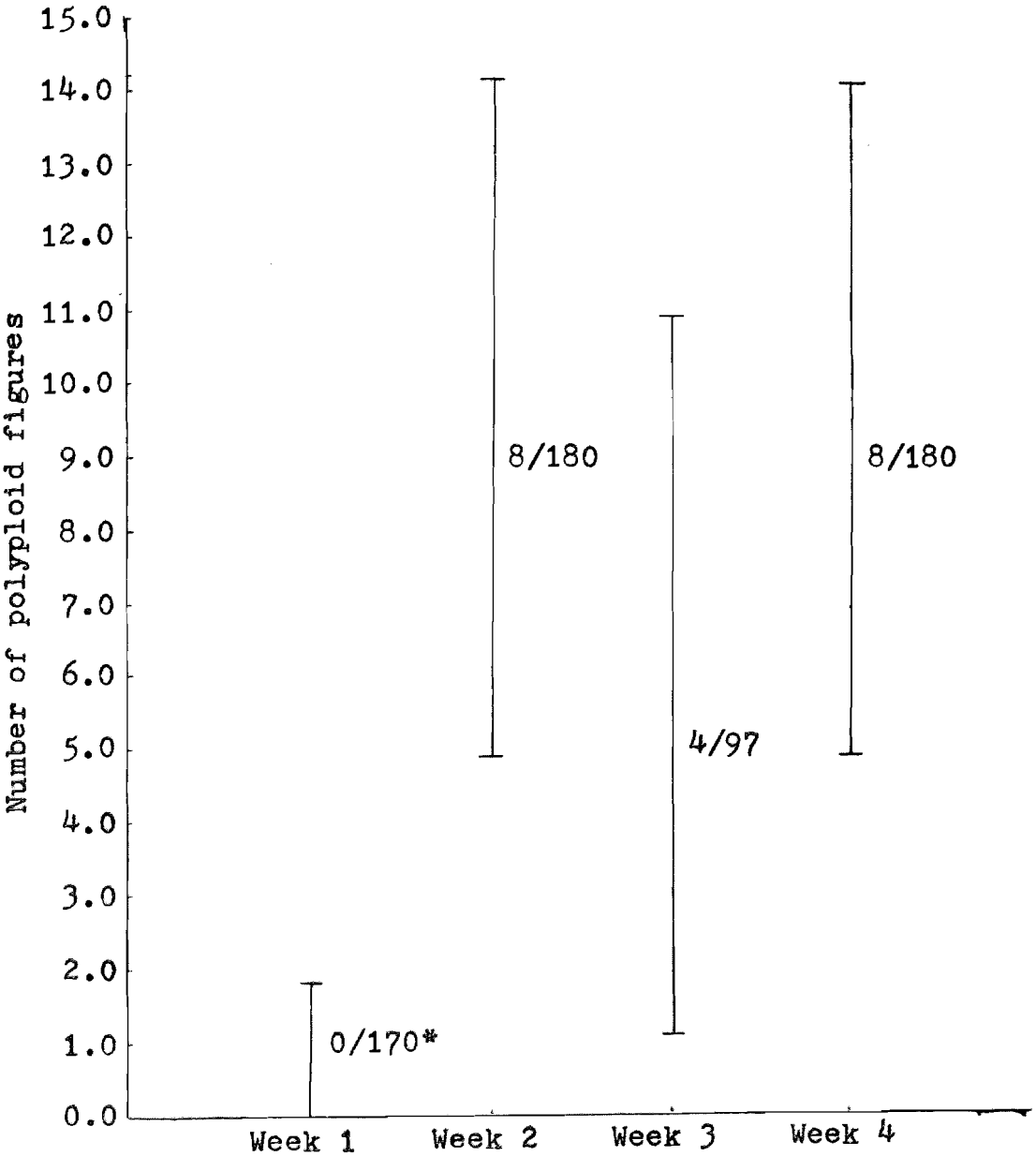


Figure 1. Average and 95% Confidence Limits of polyploidy demonstrated in metaphase figures of BALB/c mice spleen cells with a 10^{-1} Friend virus dilution during a four week period.

*Ratio of polyploid figures to total number of mitotic figures counted.

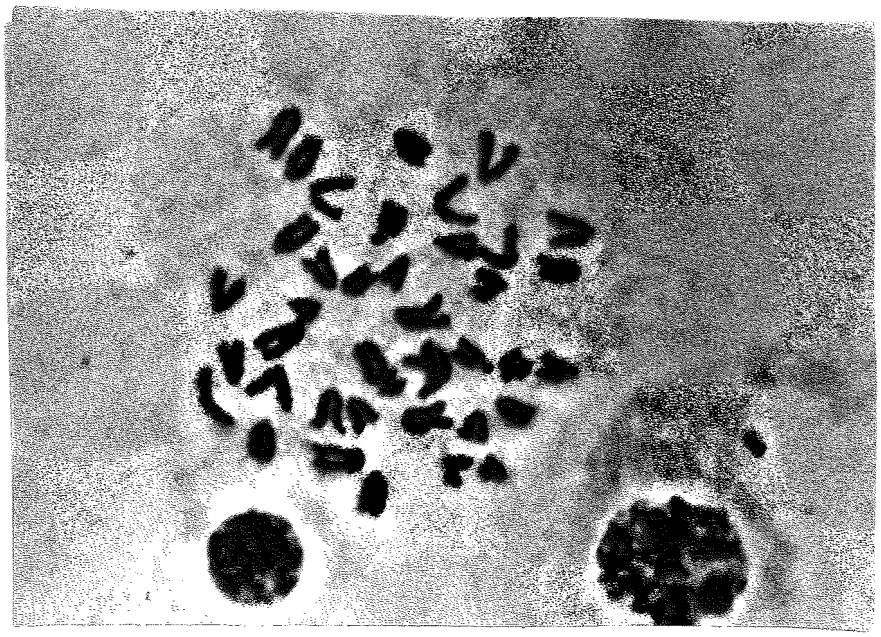


Plate 2a. Metaphase figure of a spleen cell with 41 chromosomes from a 10^{-5} dilution of Friend virus infected animal in week 1.

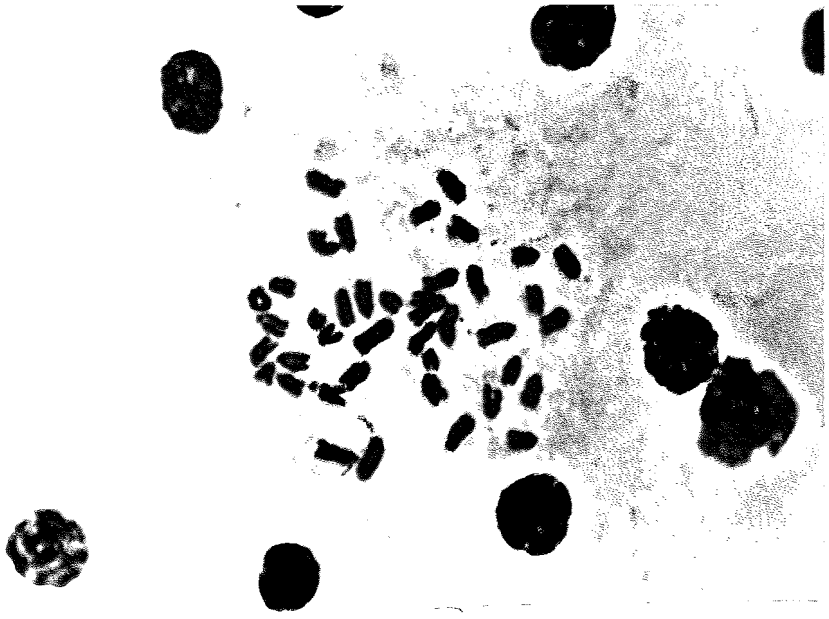


Plate 2b. Metaphase figure of a spleen cell with 41 chromosomes from a 10^{-5} dilution of Friend virus infected animal in week 4.

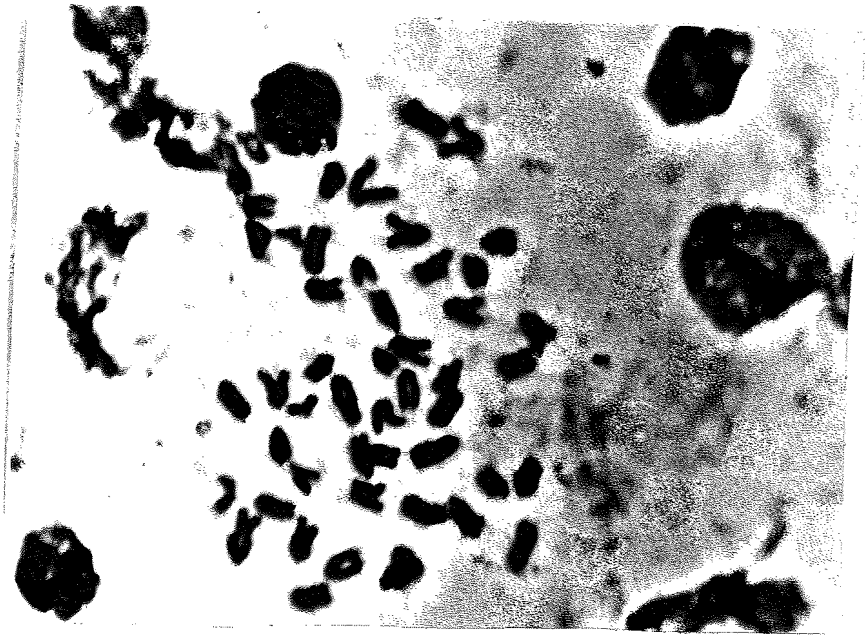


Plate 3. Metaphase figure of a spleen cell with 49 chromosomes from a 10^{-3} dilution of Friend virus infected animal in week 2.

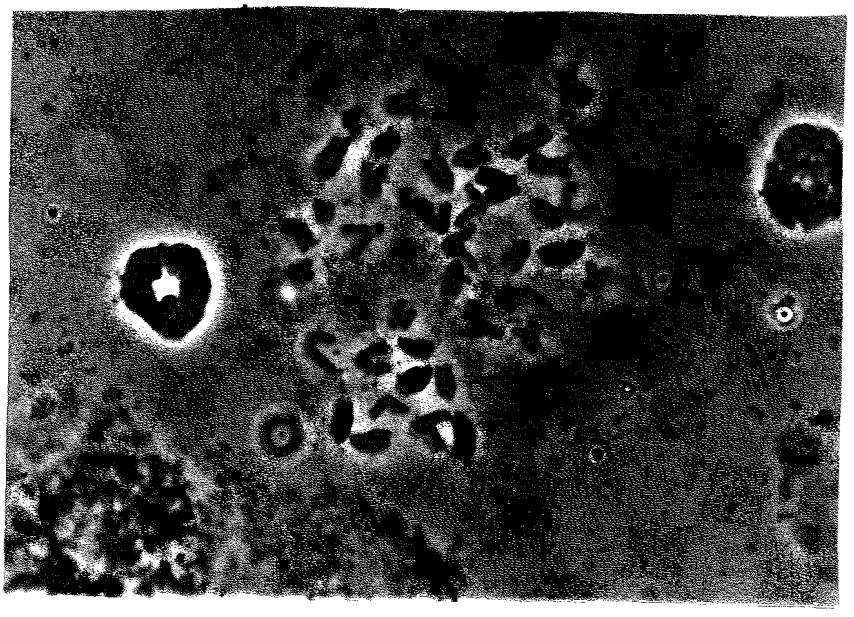


Plate 4. Metaphase figure of a spleen cell with 39 chromosomes from a 10^{-3} dilution of Friend virus infected animal in week 3. Note the typical "Friend" cell at the left of the chromosomes.

Table 1. Metaphase chromosome numbers per animal infected with the 10^{-1} Friend virus dilution.

	animal	cells counted	<37	37	38	39	40	41	>41	poly-ploid*
Week 1	1	30			2	1	27			
	2	30			2	2	24			
	3	20				2	18			
	4	30	1		1		28			
	5	30	1			2	27			
	6	30	2		2		26			
Week 2	1	30			1	1	28			
	2	30	1	1	2	1	25			
	3	30		1	1	1	23	1		3
	4	30	1			3	26			
	5	30			2	2	22			4
	6	30	1			1	27			1
Week 3**	1									
	2									
	3	23			1	4	18			
	4	30				1	25	1		3
	5	14			4		9			1
	6	30	1		5	1	23			
Week 4	1	30		1	2	2	23			2
	2	30			2	2	26			
	3	30			1	2	24			3
	4	30			2	2	24		1	1
	5	30			1	1	28			
	6	30	1		2	3	22			2
Week 5***	1	28	1	1	2	2	20	1		1
	2									
	3									
	4									
	5									
	6									

* Polyploid refers to those cells having multiples of 40, e.g. 80, 120 etc.

** Animal 1 and 2 died from ruptured spleens and proved to be inadequate for analysis. The entire set of animals appeared to be more susceptible to colchicine injections.

*** Only one animal survived for this time period from the 8 originally infected.

Table 2. Metaphase chromosome numbers per animal infected with the 10^{-3} Friend virus dilution.

	Animal	number cells counted	<37	37	38	39	40	41	>41	polyploid
Week 1	1	30			2	1	25	2		
	2	30			2	2	26			
	3	30		1	1	1	27			
	4	30		1	2	1	26			
	5	30			1	3	25			1
	6	30			3		27			
Week 2	1	30			1	2	26	1		
	2	30	1		3	1	24			1
	3	30			2	2	25	1		
	4	30		1		3	25			1
	5	30		1	3	2	24			
	6	30				1	28		1	
Week 3	1	30			2	5	23			
	2	30					27	3		
	3	30		1			29			
	4	30			1	5	24			
	5	26		1		3	22			
	6	30		3	2	1	23	1		
Week 4	1	30			2	1	26	1		
	2	30				1	27	1		1
	3	30			3	1	26			
	4	30			2		28			
	5	30		1	1	1	27			
	6	30			2		27			1
Week 5*	1	30			1	4	19	2	1	3
	2									
	3									
	4									
	5									
	6									

* Only one animal survived for this time period from the 8 originally infected.

Table 3. Metaphase chromosome numbers per animal infected with the 10^{-5} Friend virus dilution.

	animal	number cells counted	<37	37	38	39	40	41	>41	poly-ploid
Week 1	1	18	1	1	1	2	13			
	2	30	1		2	2	25			
	3	20			2		18			
	4	30			2	2	25	1		
	5	30		2	2	3	22			1
	6	30	1		2	2	25			
Week 2	1	30		1	2	2	25			
	2	30				3	27			
	3	30	1		1	3	25			
	4	25		1	2	1	21			
	5	30			1	2	26			1
	6	25	1		2	2	20			
Week 3	1	31		2	4	3	21			1
	2	15	1	1		2	11			
	3	30		1	4	1	23			1
	4	8		3	2		3			
	5	24	1	1	4	1	16			1
	6	30	1	1	2	2	24			
Week 4	1	30	1	1	1	2	25			
	2	30			1	4	23			2
	3	30		2			25	1		2
	4	30	2		1	2	24	1		
	5	30			2		28			
	6	30				1	27	1		1
Week 5*	1									
	2									
	3	30			2	1	27			1
	4	17			1		15			1
	5	30			2		27	1		
	6	30	1	1	2	1	22	1		2

* Animal 1 and 2 died from ruptured spleens and proved to be inadequate for analysis.

dilutions 10^{-1}, 10^{-3} and 10^{-5} respectively. Animals 1 and 2 in week 3 and animals 1 and 2 of week 5 died shortly after inoculation with colchicine. The missing animals 2 through 6 in 10^{-1} and 10^{-3} dilutions of virus in week 5 succumbed to the effects of the disease before the date of sacrifice. Table 4 shows the results of untreated control animals with and without colchicine treatment. No significant difference was found between these control groups. Plates 5a and 5b exhibit the appearance of normal numbers of chromosomes as they appear in untreated animals. Table 5 summarizes the percentages of cells demonstrating normal and abnormal mouse metaphase chromosome numbers during the infective stages of the disease.

Of the total number of mitotic figures examined for each mouse, the fraction (p) was determined for those appearing as a normal diploid number of 40. A constant of 0.01 was added to each p to remove zero values, then transformed to θ = arcsine value of $\sqrt{p+0.01}$. Example: at dose 10^{-1}, week 1, mouse 1, 30 figures were examined. Of these, 13 or 43.3% were normal diploid cells of 40 chromosomes containing no constrictions: θ = arcsine $\sqrt{p + 0.01}$ = arcsine $\sqrt{44.3}$ = 41.73. Because of an asymmetrical distribution of binomial proportions over most of their range, an index that is more nearly additive and has an approximately symmetrical and near-normal distribution is obtained by converting percentages to equivalent angles by an arcsine table. Table 6 gives the average arcsine transformed values of the proportions of

Table 4. Distribution of metaphase chromosome numbers and average number of constrictions of uninfected control BALB/c mice with and without colchicine.

	animal number	<39	39	40	41	average number of secondary constrictions
with Colchicine	1		2	8		0.60
	2	1	1	8		1.60
	3	1	2	6	1	1.10
without Colchicine	1	1	1	8		1.10
	2		1	9		0.70
	3		1	8	1	1.40

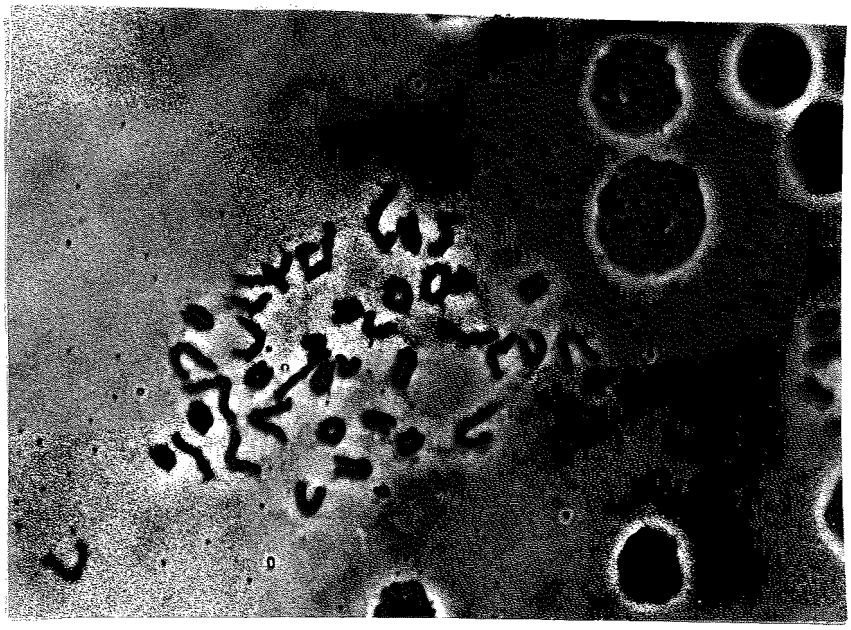


Plate 5a. Normal diploid metaphase figure of a spleen cell with 40 chromosomes from an uninfected BALB/c mouse. This was from a colchicine treated, control animal.

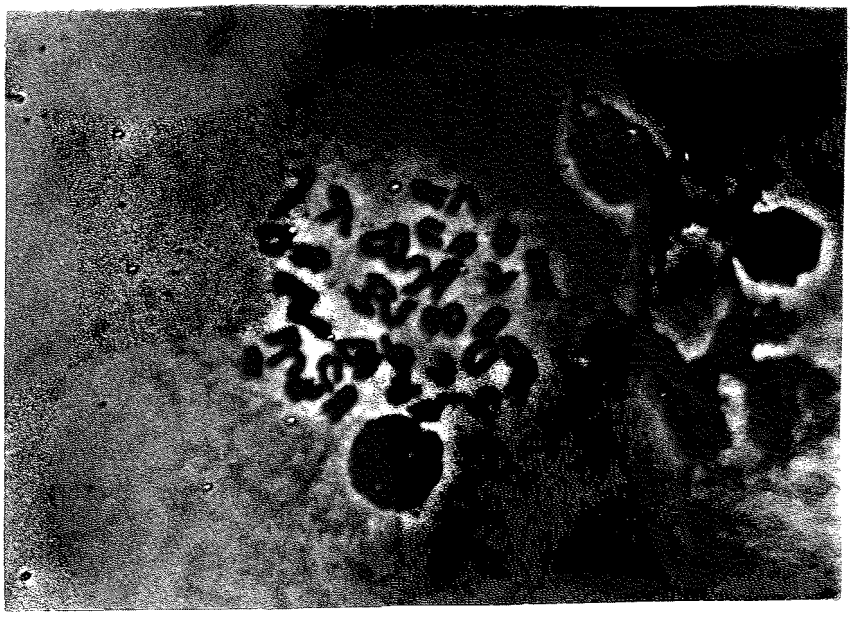


Plate 5b. Normal diploid metaphase figure of a spleen cell with 40 chromosomes from an uninfected BALB/c mouse. This was from an animal not treated with colchicine.

Table 5. Distribution of metaphase chromosome numbers in BALB/c mice with various dilutions of Friend virus.

	figures counted	<39	39	40	41	>41	poly-ploid
controls	60	5%	13.3%	78.3%	3.3%	---	0.0%
10^{-1}	651	7.5%	5.5%	83.1%	0.4%	0.1%	3.2%
10^{-3}	746	6.3%	5.5%	85.2%	1.7%	0.3%	1.0%
10^{-5}	753	10.2%	5.8%	81.4%	0.7%	---	1.7%

Table 6. Average arcsine transformed values of the proportion of normal diploid chromosome patterns among spleen cells examined from BALB/c mice held up to 5 weeks following 3 dose levels of Friend virus. 95% Confidence Limits are shown in parentheses.

Virus Dilution	Week 1	Week 2	Week 3	Week 4	Week 5
10^{-1} Averages	37.6 (±6.08)	18.4 (±6.08)	12.3 (±7.46)	9.5 (±6.08)	5.7 (±14.9)
10^{-3} Averages	43.6 (±6.08)	27.1 (±6.08)	12.8 (±6.08)	12.0 (±6.08)	5.7 (±14.9)
10^{-5} Averages	40.8 (±6.08)	35.4 (±6.08)	17.9 (±6.08)	19.1 (±6.08)	12.3 (±7.46)

Normal controls: 48.79

normal diploid chromosome patterns. Table 7 summarizes the proportion of normal mitotic patterns of diploid cells from spleen cells of mice held up to 5 weeks after receiving 3 doses of Friend virus. As seen in Figure 2, weeks 1, 3, 4, and 5 showed no significant difference with respect to dose of virus. The interaction between dose and week failed to be significant also. Only at week 2 did the proportion of normal cells differ significantly between the three virus dilutions and only then between the highest and lowest doses of virus. The proportion of cell configurations showing abnormal numbers differed significantly from non-virus-infected animals after week 1.

Chromosome Aberration

Each metaphase figure was carefully examined for the presence of chromosome aberrations. Achromatic zones or secondary constrictions were noted near the centromere on many of the chromosomes. The average number of constrictions per cell among those cells containing constrictions was obtained for each animal where data permitted. Those cells with abnormal chromosome counts were included within the analysis; however, those that contained no constrictions were ignored. Plates 6a and 6b demonstrate the occurrence of secondary constrictions noted in the analysis. In addition, some cells exhibited a primary constriction along the arm or

Table 7. Summary of analysis of variance of the proportion of normal mitotic patterns of diploid cells among those cells examined from spleen cells of mice held up to 5 weeks after receiving 3 doses of Friend virus.

Source	df	SS	MS	F
Total	75	14,497.207		
Treatments	14	11,100.932		
Dose	2	540.174	270.087	4.85*
Weeks	4	9,666.692	2,416.673	43.41**
Dose x Weeks	8	894.066	111.758	2.01
Error	61	3,396.275	55.677	

$\sqrt{EMS} = 7.46$

* significance at p = 0.05
** significance at p = 0.01

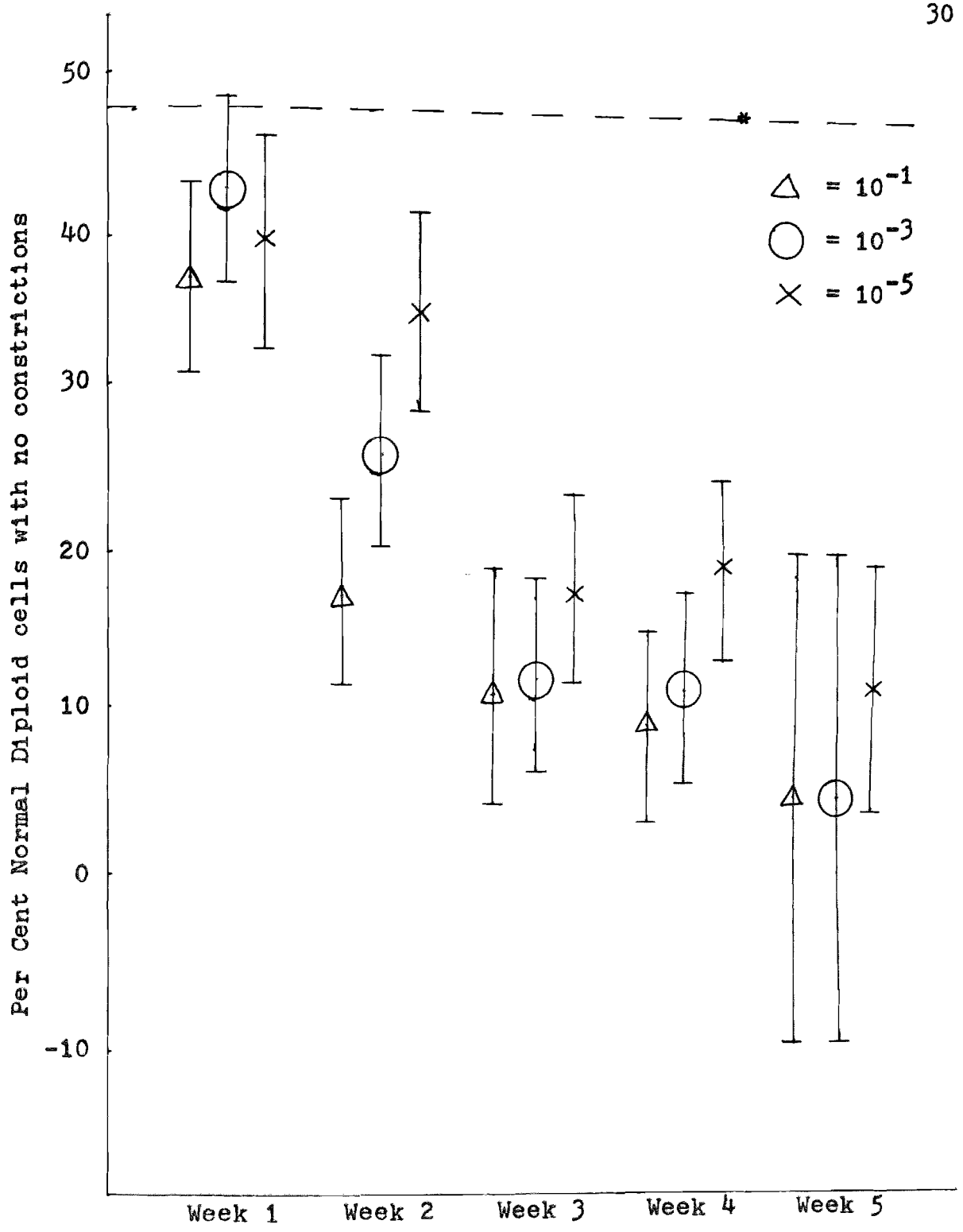


Figure 2. Proportion of normal diploid cell patterns among spleen cells at metaphase from BALB/c mice held up to 5 weeks after inoculation of 3 levels of Friend virus. Average value and 95% Confidence Limits shown.

* Normal controls (no virus)

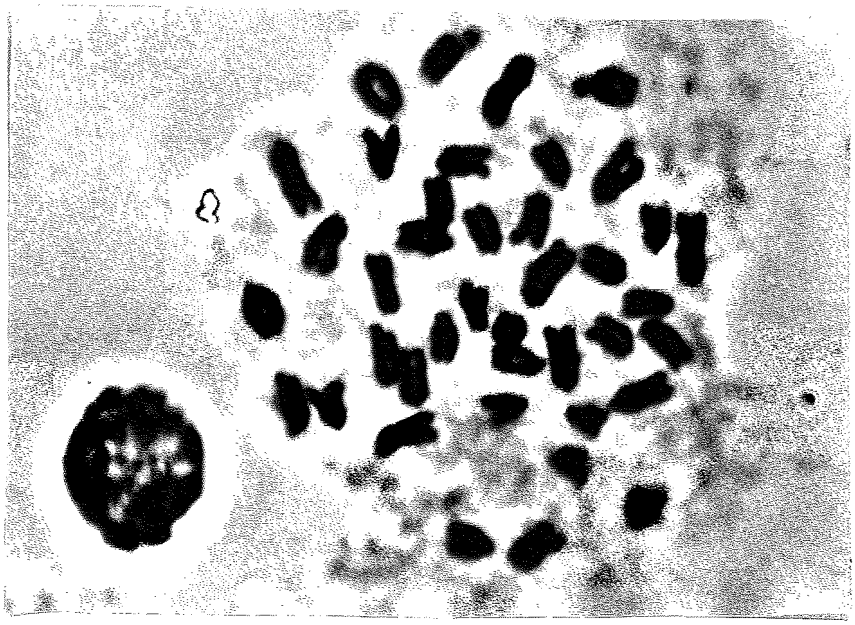


Plate 6a. Diploid metaphase figure of a spleen cell with 40 chromosomes demonstrating 2 secondary constrictions from 10^{-1} dilution Friend virus infected animal in week 1.

Plate 6b. Diploid metaphase figure of a spleen cell with 40 chromosomes demonstrating 4 secondary constrictions from 10^{-3} dilution Friend virus infected animal in week 1.

arms of the chromosome as is demonstrated in Plate 7. These were considered in with the average number of constrictions along with the secondary constrictions with no separation of the two in the analysis.

To determine the influence of colchicine on the phenomenon of secondary constrictions, metaphase plates in animals without colchicine pretreatment were studied. No significant effect on the incidence of secondary constrictions of colchicine treated uninfected animals were noted so that animals with and without colchicine treatment were analyzed as control animals (Table 4).

Other instances of abnormalities also appeared; however, they appeared in such small numbers that only mention of them will be made. Plate 8 shows a karyotype of a spleen cell demonstrating a round or ring chromosome which was seen only a few times during the course of the experiment. Some appearances of long or short arms were noted, but it was difficult to determine whether it was an actual representation of the chromosome structure and form.

Figure 3 shows the average number of constrictions per cell which demonstrated constrictions. Considering the 10^{-1} virus dose, the 99% confidence level for the average number of constrictions differed significantly at week 1 from weeks 2 and 4. Over all, virus doses 10^{-1} and 10^{-3} do not differ significantly with respect to the average number of constrictions. The average number of constrictions found at the 10^{-5}

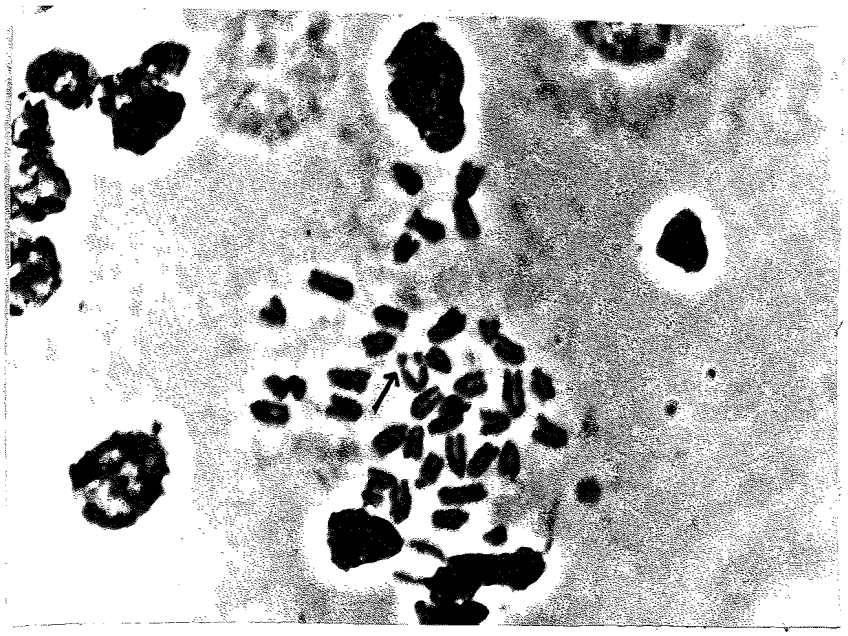


Plate 7. Diploid metaphase figure of a spleen cell with 40 chromosomes from 10^{-3} dilution Friend virus infected animal in week 3. Note the constrictions shown by the arrow.

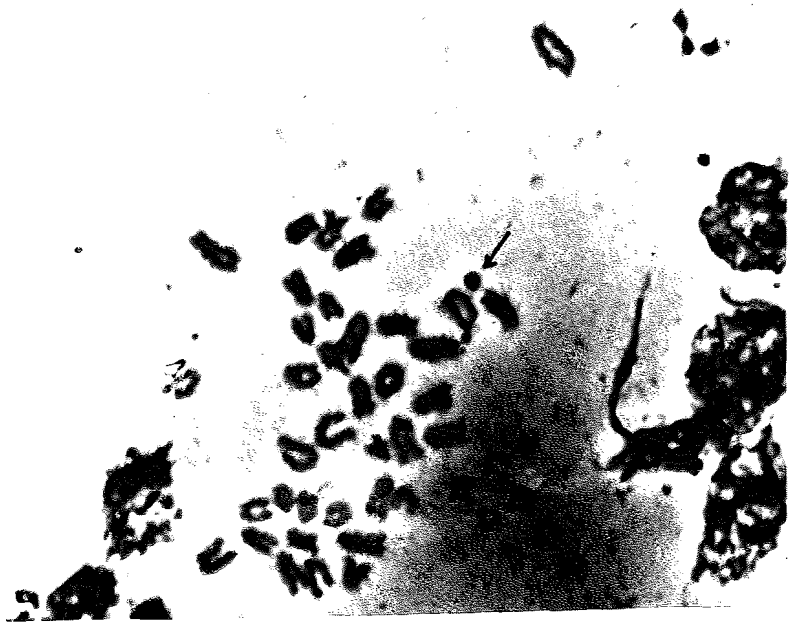


Plate 8. Diploid metaphase figure of spleen cell with 40 chromosomes from a BALB/c mouse infected with a 10^{-5} Friend virus dilution infected animal in week 3. Note the ring chromosome shown by the arrow.

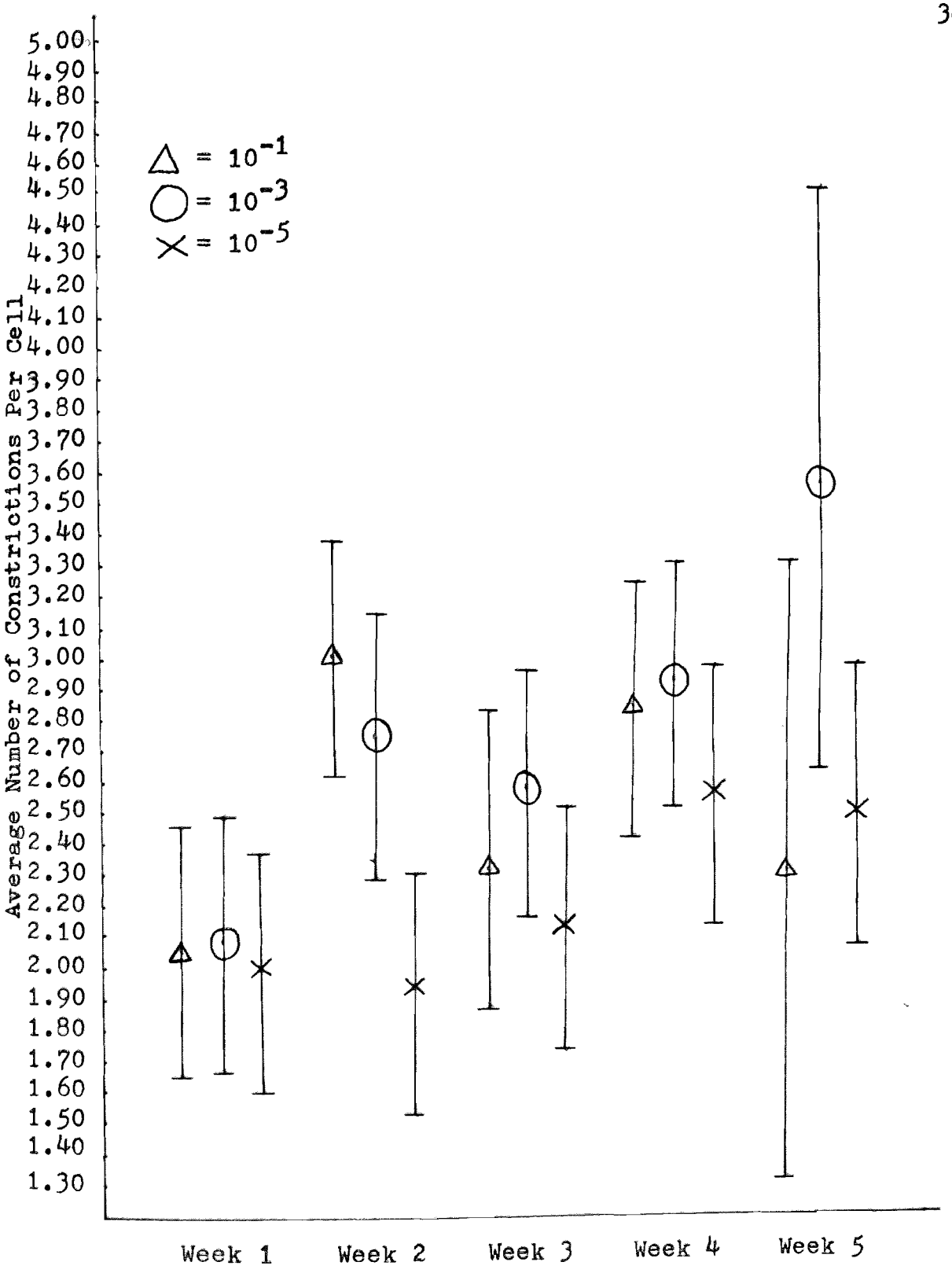


Figure 3. Average number of constrictions per cell among those containing chromosome constrictions of BALB/c mice infected with three doses of Friend virus. The class widths for the 99% Confidence Limits are also shown.

dose is significantly lower than the 10^{-1} dose in week 2. Overlap occurred in all five weeks in the 10^{-3} and 10^{-5} virus doses.

Spleen Weights

Spleen weights were analyzed without taking into account the body weights or the growth of the mice in the 5-week period. Normal spleen weights differ so little from week to week over a considerable period of time in the adult stage of the mouse that it was not necessary to take it into account.

Raw spleen weights were found to be unsuitable for analysis so a transformation was necessary to fulfill the assumptions underlying the analysis of variance techniques. For the analysis which is summarized in Table 8, the transformed spleen weights ($\sqrt{\text{spln. wt.}}$) was used and a 5 x 3 completely randomized 2-way design applied. As Table 8 shows, both week and virus dilution effects were significant as was their 2-way interaction. Figure 4 represents the average transformed spleen weight with the 99% confidence class limits included. There is a significant difference demonstrated here between 10^{-1} and 10^{-5} viral dilutions after week 1 as the spleen weights increase over the 5-week infective period. The overlap of 10^{-1} and 10^{-3} dilutions demonstrates their similar infectivity during the course of the disease. At week 5, only one animal survived the effects of the disease thereby attributing the wide range demonstrated. Figure 4

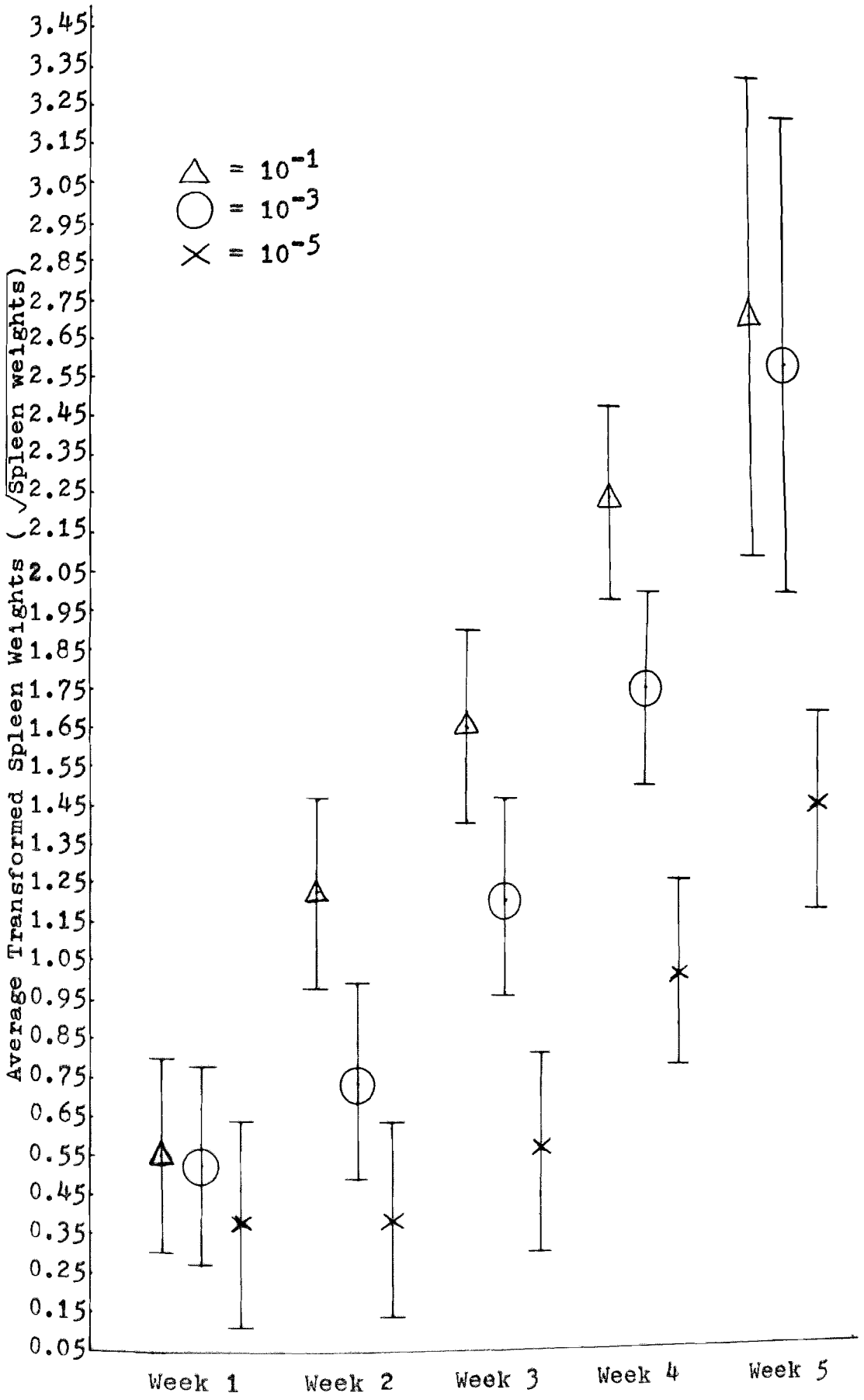
Table 8. Summary of analysis of variance of spleen weights of BALB/c mice treated with 3 doses of Friend virus over a 5-week infection period.

Source	df	SS	MS
Total	79	34.4470004875	
Treatments	14	30.8781256535	2.20558
Dose	2	6.9461960408	3.473098**
Weeks	4	18.0014120569	4.50035**
Dose x weeks	8	5.93051755580	0.74131469
Error	65	3.568874834	0.0549058

$\sqrt{EMS} = 0.2343198$

** p = 0.01

Figure 4. Average transformed spleen weights of BALB/c mice held up to 5 weeks following administration of 3 doses of Friend virus. The class widths for the 99% Confidence Limits are also shown.


Average Transformed Spleen Weights (√Spleen weights)
3.45
3.35
3.25
3.15
3.05
2.95
2.85
2.75
2.65
2.55
2.45
2.35
2.25
2.15
2.05
1.95
1.85
1.75
1.65
1.55
1.45
1.35
1.25
1.15
1.05
0.95
0.85
0.75
0.65
0.55
0.45
0.35
0.25
0.15
0.05
△ = 10⁻¹
○ = 10⁻³
× = 10⁻⁵
Week 1
Week 2
Week 3
Week 4
Week 5

indicates a good correlation between the spleen weights of animals infected with either the 10^{-1} or 10^{-3} virus dose during the entire experiment. Once the disease is initiated, the spleen weights increase approximately linearly throughout the 5-week course of these experiments.

A correlation of spleen weights and the average number of constrictions per metaphase figure in those cells demonstrating constrictions involved a bivariate correlation routine found in Sokal and Rohlf, (1969). Figure 5 demonstrates the significance by which the two variables Y_1, spleen weights transformed to $\sqrt{x + 0.5}$, and Y_2, average number of constrictions per figure, correlate in a population ellipse at the 95% confidence level. Using the correlation coefficient (r) as 0.53543, which according to analyses which are appropriate for bivariate analysis, the value t is significantly greater than zero as calculated by $t_{.05/n-2} = r / \sqrt{(1-r^2)/ n-2} \cong 7$. This value is greater than the critical values calculated for $t_{.05/n-2} \cong 2.00$ or $t_{.01/n-2} \cong 2.66$.

A similar correlation was carried out using the variables Y_1 as the spleen weight transformed to $\sqrt{x + 0.5}$ and Y_2 as the arcsine $\sqrt{p + 0.01}$ where p is the proportion of normal cells among those examined. Figure 6 exhibits the plotted points of the 95% confidence ellipse demonstrating the strong and significant correlation coefficient of these two variants.

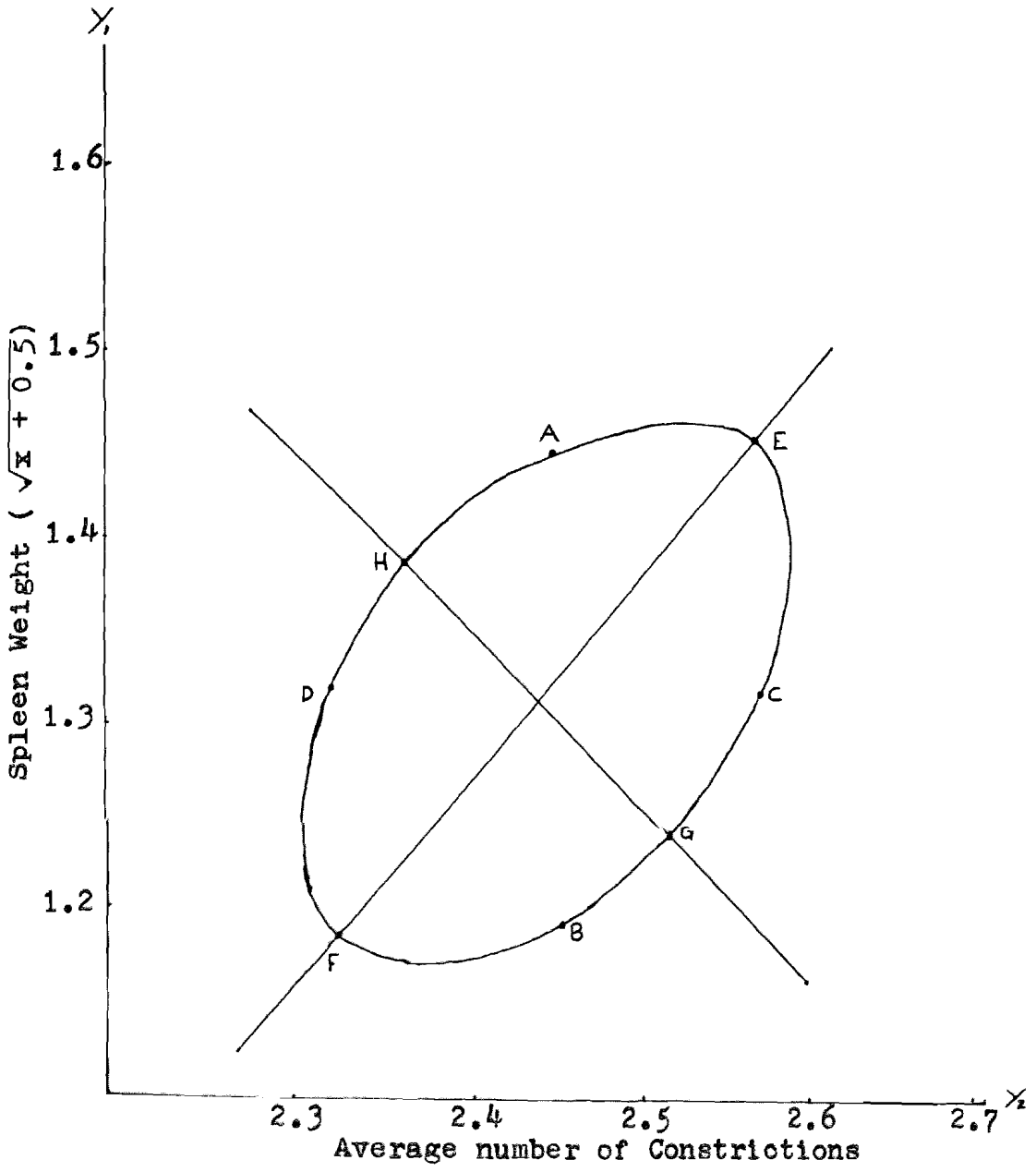


Figure 5. Correlation of spleen weights and average number of chromosomal constrictions in metaphase figures of spleen cells containing chromosomal constrictions during Friend virus leukemia of BALB/c mice.

Figure 6. Correlation of spleen weights to proportion of normal diploid cells among mitotic figures examined during Friend virus leukemia of BALB/c mice.

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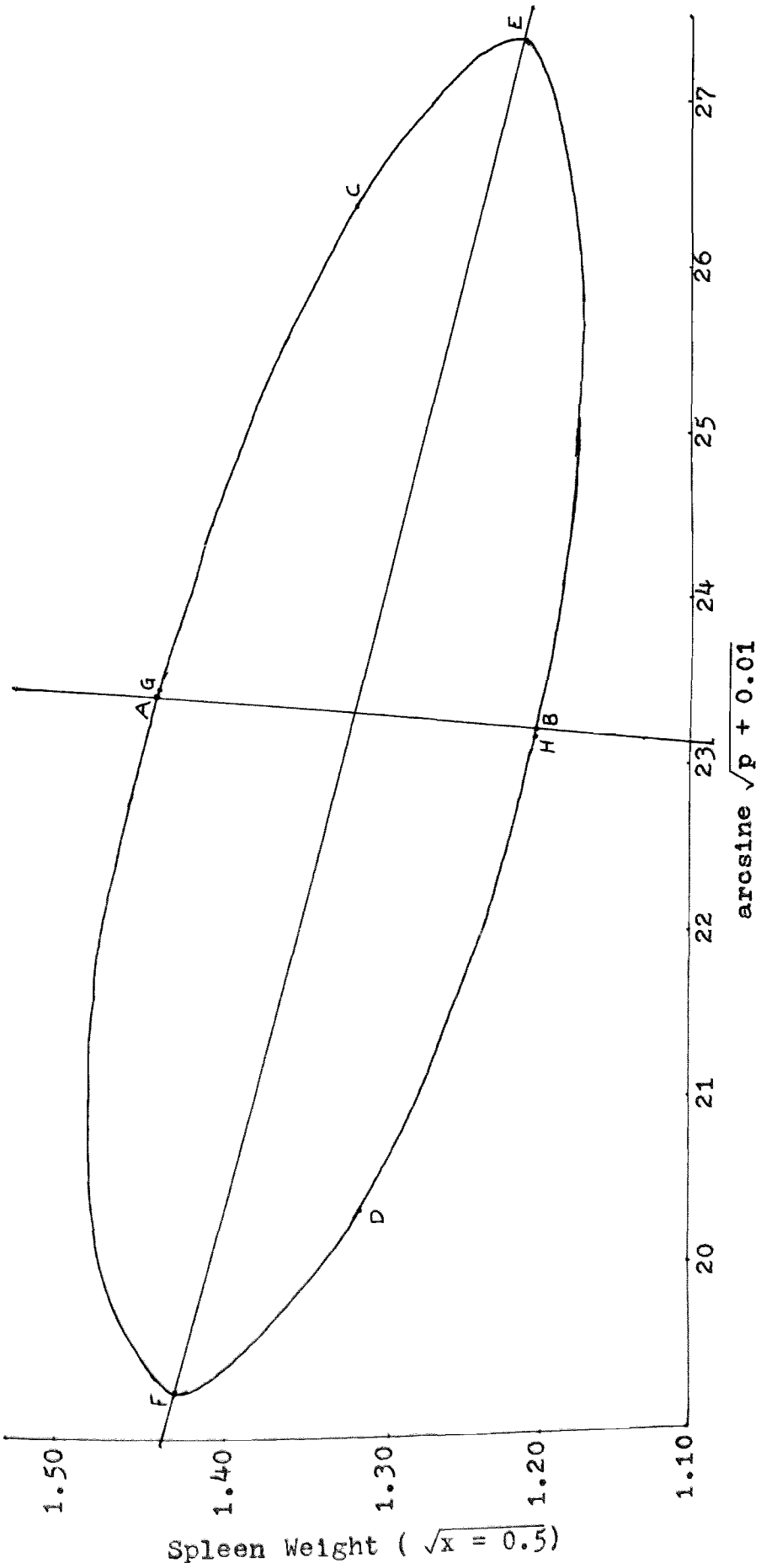
d during



DISCUSSION

The metaphase figures used for these chromosome studies of infected mice were induced by three intraperitoneal doses (10^{-1}, 10^{-3}, and 10^{-5}) of Friend virus into BALB/c mice. Untreated animals that received no virus were also examined. Splenomegaly developed after a relatively short induction period with each dose of virus. This controlled sequential development of the Friend virus leukemia made it suitable for comparing the chromosomal changes in the progression of the disease.

Metaphase figures with an altered chromosome number were routinely found in the spleen cells of infected animals. Some observed changes were multiples of the normal diploid number of 40, the appearance of figures with 41 chromosomes and cells containing numbers of 42, 44 and 49 chromosomes. In addition, metaphase figures with less than 40 chromosomes were noted throughout the experiment. These included numbers of 39, 38 and 37 with a few figures with less than 37 chromosomes. The abnormal chromosome numbers in infected animals, with the exception of polyploidy, were noted with the same approximate numbers as found in uninfected animals.

The appearance of polyploid cells is intimately associated with the process of leukemogenesis since they were completely absent in the uninfected animals. In the 10^{-1} dilution of Friend virus, the number of polyploid cells found in weeks 2 and 4 is significantly different from

control animals. A statistically significant increase at the 95% confidence level occurred in the number of polyploid animals in weeks 2, 3 and 4 when compared to normal animals. Week 5 failed to give substantial information because only a single animal survived the leukemic disease at the 10^{-1} virus dilution. The number of metaphase figures counted for each dilution is approximately the same, therefore it can be considered that the 10^{-1} dose of Friend virus has an effect upon the incidence of polyploid figures. The 10^{-3} and 10^{-5} dilutions of Friend virus demonstrate the polyploid figures but to a much lesser degree per total number of figures than the 10^{-1} virus dilution. The failure of polyploid cells to occur even with a massive 10^{-1} dose of virus until the second week of the disease would in all probability exclude this cell abnormality as a cause of the Friend virus leukemia.

The chromosome number of 41 was never found in our experiments with any virus dose to be near the figure of 9% reported by Wakonig-Vaartaga (1960). The report was based on no more than one animal infected with an unreported dose of Friend virus. Also, a time-interval study was not conducted to determine the sequential incidence of the reported chromosome number of 41.

The proportion of normal diploid cell configurations of infected animals differs significantly from normal uninfected animals after week 1 (Figure 2). In week 1, only the 10^{-3} virus dose overlapped the proportion of normal diploid

cell configurations of uninfected controls and then only slightly. All other weeks and virus doses were significantly lower in the proportion of normal diploid cell figures than the control animals. Week 2 was the only week showing a difference between the virus doses, and then only between the lowest and highest doses. The overlap between doses which generally occurred showed that once the proliferation of cells had begun the dosage level had an insignificant action. However, the time interval showed a regression of normal diploid cell configurations, especially in the 10^{-1} and 10^{-3} Friend virus dilutions.

Bayreuther (1960) reported that marked predominance of normal chromosome complements were found in representative material from precancerous stages, benign tumors, and in the majority of early malignant tumors, both spontaneous and induced by virus. He also demonstrated that the genetic variation at the chromosome level need not necessarily precede the malignant change. Wakonig and Stich (1960) concluded that neoplastic cells can have a diploid chromosome number with no detectable chromosome abnormalities. Doubt is expressed concerning the malignant nature of diploid cell, but transplantation of cytologically normal cells resulted in rapid formation of tumors, a general leukemia, or both, without any visible alteration of normal chromosome complements. Our data differ from that reported by the above authors in that a decrease of normal diploid cells appeared as the disease progressed through a five-week

course of study (Figure 2). Especially noteworthy in our findings is the decreased number of cells with a normal chromosome complement after week 1.

Wakonig-Vaartaja (1960) considers aneuploidy, as a result of neoplasia, to be a secondary phenomenon in the growth of these cell populations. The decreased proportion of normal diploid cells patterns and almost linear increase of the average number of constrictions per cell demonstrating chromosome constrictions in our results appears to contrast with this observation. However, it would be presumptive to suggest that aneuploidy and increased number of constrictions are a cause of the leukemic disease. Nowell, et al. (1964) observed clones of cells with extensive chromosome changes persisting in hematopoietic tissues for long periods after irradiation without leukemia arising and an inconsistency of chromosome changes from case to case. The lack of consistency and absence of leukemia when chromosome changes appear suggest that they are epiphenomea, either unrelated to the leukemia or contributing to the progression of the leukemia, but not the cause of the neoplastic state. Both "balanced" abnormalities (one abnormally long chromosome and one abnormally short chromosome) and "unbalanced" abnormalities (two stem lines with 41 chromosomes and one stem line with 75 chromosomes) were found, yet no gross or microscopic evidence of leukemia was observed (Nowell et al., 1964). Although no examination of stem lines was carried out in the present investigation, the consistency of decreased

normal diploid cell patterns and increased average number of constrictions suggest that these alterations may be involved in the induction of Friend virus disease and not just a result of the disease.

The observed intense spleen cell proliferation coincides with the appearance of increased numbers of chromosomes with secondary constrictions. Secondary constrictions were noted near the centromere in some chromosomes of all mitotic figures in infected BALB/c mice. In all weeks, infected animals showed a higher number of constrictions than the normal animals. The average number of constrictions in infected animals differed significantly at week 2 between the 10^{-1} and 10^{-5} virus dilutions. A very slight overlap occurred between 10^{-3} and 10^{-5} at week 2, and the remaining weeks showed a strong overlap of all three doses. The wide ranges demonstrated in 10^{-1} and 10^{-3} dilutions of virus in week 5 were caused by loss of animals due to the effects of the disease. If a secondary constriction is indeed a chromosomal aberration, our data again differ from that reported by Bayreuther (1960) and Wakonig and Stich (1960) in that we found a statistically significant increased number of secondary constrictions beginning with the first week of the disease.

Polyploid figures demonstrated approximately the same number of secondary constrictions and in the same locations as did the normal diploid figures of infected animals. Tsuchida and Rich (1964) reported cells containing a

greater-than-diploid chromosome number rarely contained more than one distinct secondary constriction. The number of polyploid cells found in this study were not reported by the above authors. The polyploid patterns observed are probably the expected result of an aberrant form of cell division in those diploid cells already demonstrating secondary constrictions previous cell division. Minute or small chromosomes arising through secondary constriction of existing chromosome pairs (Tsuchida and Rich, 1964) were also not observed in our experiments.

A significant difference in spleen weights was observed in animals receiving a 10^{-1} or 10^{-3} Friend virus dilution. After the first week, the spleen weights increased linearly with respect to each dilution demonstrating that the number of infective particles inoculated into BALB/c mice are directly related to splenomegaly. Slight overlap occurs between 10^{-1} and 10^{-3} virus doses in week 5.

A pronounced correlation was observed between spleen weight and the average number of cells containing constriction. A significant correlation was also demonstrated between spleen weights and the proportion of normal diploid cell configurations of mitotic figures. These relationships suggest that as the disease process progresses (as manifested by the increase in spleen weights) the number of spleen cells showing the expected number of secondary constrictions increases and the proportion of normal diploid cells among mitotic figures is decreased. This may be expected since histologically

the spleen becomes markedly infiltrated with tumor cells as the Friend disease progresses. The increased incidence of constrictions in this study corresponds to the splenic hyperplasia period of Friend virus and not to the leukemia period as reported by Tsuchida and Rich (1964). However, the aberrations associated with the Friend virus in this study do not demonstrate the biphasic response that these authors observed.

Zuelzer and Cox (1969) state that the apparent association of acute leukemia with a variety of chromosomal disorders of genetic origin suggest that the genetic mechanisms form part of the causal chain of leukemogenesis in a small minority of human leukemia cases. In the murine leukemias "a complex interaction among host factors and extraneous factors determines the incidence, latency and hematologic type" (Upton et al., 1965). Interactions involving viruses might be variable according to the mitotic rate of a given cell population, the timing of the exposure in relation to age, immunity or lack thereof, and dosage effects for infection. In our experiments, we controlled the dose of virus, age of exposure, and used a strain of mice 100% susceptible to Friend virus. We demonstrated that early in the Friend virus disease at any virus dose used that a statistically significant increase in the number of secondary constrictions occurred. Also, a reduction in the proportion of normal diploid cells among mitotic figures was demonstrated during the five weeks of the experiment.

The question is this: did our observed abnormalities really cause or result from the disease? Since the abnormalities occurred relatively early in the disease and even with a very low dose of virus, it does not seem likely that the observed abnormalities were only a secondary phenomenon of the disease. Further work in a more controlled environment is needed. Experiments are planned in which tissue culture cells of a given hematological type will be examined for changes in the chromosome complement and abnormalities in chromosomes after Friend virus infection.

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